(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 August 2001 (09.08.2001)

PCT

(10) International Publication Number
WO 01/57183 A3

(51) International Patent Classification[7]: C07K 1/04, C12P 21/02, 21/04, 21/06, C12N 9/00

(21) International Application Number: PCT/US01/03147

(22) International Filing Date: 31 January 2001 (31.01.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/180,319 4 February 2000 (04.02.2000) US

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(81) Designated States *(national)*: JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *with international search report*

(88) Date of publication of the international search report:
14 February 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR PRODUCING CIRCULAR OR MULTIMERIC PROTEIN SPECIES *IN VIVO* OR *IN VITRO* AND RELATED METHODS

(57) Abstract: A method is disclosed for the *in vitro* or *in vivo* cyclization of protein or peptide sequences. Also disclosed is a method of fusing polypeptide sequences while bound to a solid support. These protein manipulation techniques relied on the *trans*-splicing activity of a split intein, such as the naturally occuring split intein form the *dnaE* gene of *Synechocystis* sp. PCC6803 (*Ssp* DnaE intein). The cyclization procedures required the fusion of C- and N-terminal intein splicing domains to the N- and C-termini, respectively, of a target protein (Intein$_C$-target protein-Intein$_N$). Cyclization *in vivo* occurred post-translationally when the two complementary intein splicing domains ligated the N- and C-terminus of the target protein. In vitro cyclization also utilized and Intein$_C$-target protein-Intein$_N$ precursor protein, in which the intein domains were fused to a chitin binding domain (CBD). Protein expression was conducted under conditions that favored the accumulation of precursor protein, which was immobilized on a chitin resin. The circular protein species were eluted from the chitin resin following incubation under conditions that favored protein splicing. *Trans*-splicing was used to ligate polypeptides on a solid support by generating a protein composed of a CBD fused to a C-terminal intein splicing domain and target protein (1). This was incubated with a protein composed of target protein (2) fused to an N-terminal intein splicing domain and a CBD. The precursor proteins were immobilized on a chitin resin where *trans*-splicing resulted in the ligation of target protein (1) to target protein (2). These techniques greatly expand the procedures available for protein engineering and modification.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/03147

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07K 1/04; C12P 21/02, 21/04; 21/06; C12N 9/00
US CL : 435/68.1, 69.1, 69.7, 183; 530/334, 413
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/68.1, 69.1, 69.7, 183; 530/333, 334, 413

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHONG et al. Utilizing the C-terminal cleavage activity of a protein splicing element to purify recombinant proteins in a single chromatographic step. Nucleic acids research (ENGLAND) 15 November 1998 (15.11.1998), Vol. 26, No. 22, pages 5109-5115, entire document.	1-18
Y	EVANS et al. Semisynthesis of cytotoxic proteins using a modified protein splicing element. Protein science (UNITED STATES) November 1998 (11.1998). Vol. 7, No. 11, p2256-2264, entire document.	1-10
X — Y	EVANS et al. Intein-mediated protein ligation: harnessing nature's escape artists. Biopolymers (UNITED STATES) 1999, Vol. 51, No. 5, pages 333-342, especially figure 5.	12 — 1-11, 13-18
Y	MATHYS et al. Characterization of a self-splicing mini- intein and its conversion into autocatalytic N- and C-terminal cleavage elements: facile production of protein building blocks for protein ligation. Gene (NETHERLANDS) 29 April 1999 (29.04.1998). Vol. 231, No. 1-2, pages 1-13, entire document.	1-18
X	US 5834247 A (COMB et al.) 10 November 1998 (10.11.1998) entire document, especially Claims 46-103.	1-18
X	SCOTT et al. Production of cyclic peptides and proteins in vivo. Proceedings of the National Academy of Sciences of the United States of America (UNITED STATES) 23 November 1999 (23.11.1999) Vol. 96, No. 24, pages 13638-13643, entire document.	11-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
25 July 2001 (25.07.2001)

Date of mailing of the international search report
14 AUG 2001

Name and mailing address of the ISA/
European Patent Office
Facsimile No.

Gabriele E. Bugaisky
Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/03147

Continuation of B. FIELDS SEARCHED Item 3:
WEST files USPT, DWPI, EPAB, JPAB; DIALOG files 411, 155, 5, 34 [ONESEARCH (allscience), MEDLINE, BIOSIS, SCISEARCH

search terms: INTEIN?, INTERVEN?, INTRON?, PROTEIN?, PEPTIDE?, FUS?, CHIMER?, TRANS, SPLIC?

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

09/937070

Applicant's or agent's file reference NEB-177-PCT	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US01/03147	International filing date *(day/month/year)* 31 January 2001 (31.01.2001)	Priority date *(day/month/year)* 04 February 2000 (04.02.2000)
International Patent Classification (IPC) or national classification and IPC IPC(7): C07K 1/04; C12P 21/02, 21/04; 21/06; C12N 9/00 and US Cl.: 435/68.1, 69.1, 69.7, 183; 530/334, 413		
Applicant NEW ENGLAND BIOLABS, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of report with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05 September 2001 (05.09.2001)	23 October 2002 (23.10.2002)
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Dorithea Laurence for Gabriele E. BUGAISKY Telephone No. 708 308-0196

Form PCT/IPEA/409 (cover sheet)(July 1998)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US01/03147

I. Basis of the report

1. With regard to the **elements** of the international application:*

☒ the international application as originally filed.

☒ the description:
pages 1-24 as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

☒ the claims:
pages 25-27 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

☒ the drawings:
pages 1-6 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

☐ the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____.

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination(under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages none

☒ the claims, Nos. none

☒ the drawings, sheets/~~fig~~ none

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

*** Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US01/03147

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims NONE	YES
	Claims 1-18	NO
Inventive Step (IS)	Claims NONE	YES
	Claims 1-18	NO
Industrial Applicability (IA)	Claims 1-18	YES
	Claims NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-18 lack novelty under PCT Article 33(2) as being anticipated by US 5834247. The reference provides for proteins comprising a controllable intervening protein sequence (CIVPS) inserted into or adjacent a target protein, the CIVPS being capable of excision from or cleavage of the modified protein under predetermined conditions in cis or in trans, i.e., increase in temperature, exposure to light, unblocking of amino acid residues by dephosphorylation, treatment with chemical reagents or deglycosylation. The reference teaches the CIVPS may also be inserted into a region that substantially inactivates target protein activity. It further states that the CIVPS can be used in a number of applications including purification of the target protein in a one-step protocol.

Claim12 lacks novelty under PCT Article 33(2) as being anticipated by Scott *et al* The reference is deemed anticipatory for the claimed subject matter because is provides intein mediated production of polypeptides *in vivo*.

Claims 1-11 and 13-18 lack novelty under PCT Article 33(2) as being anticipated by Evans *et al*. (Biopolymers 1999). The reference is deemed anticipatory for the claimed subject matter because it describes a two intein system for production of peptidases.

---------- NEW CITATIONS ----------